Plant Physiol. (1992) 100, 1503-1507
0032-0889/92/100/1503/05/$01.00/0

Received for publication April 30, 1992
Accepted July 10, 1992

Expression of a Maize Ubiquitin Gene Promoter-*bar* Chimeric Gene in Transgenic Rice Plants[1]

Seiichi Toki, Susumu Takamatsu, Chyuhei Nojiri, Shinya Ooba, Hiroyuki Anzai, Michiaki Iwata, Alan H. Christensen[2], Peter H. Quail, and Hirofumi Uchimiya*

Department of Botany, Faculty of Science, Hokkaido University, Sapporo 060, Japan (Se.T.); Fukui Agricultural Experiment Station, Fukui City 910, Japan (Su.T.); Meiji Seika Ltd. Pharmaceutical Research Center, Yokohama 222, Japan (C.N., H.A., M.I.); Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan (S.O., H.U.); and University of California, Berkeley/United States Department of Agriculture Plant Gene Expression Center, Albany, California 94710 (A.H.C., P.H.Q.)

ABSTRACT

We have constructed a chimeric gene consisting of the promoter, first exon, and first intron of a maize ubiquitin gene (*Ubi-1*) and the coding sequence of the *bar* gene from *Streptomyces hygroscopicus*. This construct was transferred into rice (*Oryza sativa* L.) protoplasts via electroporation, and 10 plants were regenerated from calli that had been selected for resistance to exogenously supplied bialaphos. Transgenic plants grown in a greenhouse were resistant to both bialaphos and phosphinothricine at a dosage lethal to untransformed control plants. Evidence of stable integration of the transferred gene into the genome of the regenerated primary transformant plants was obtained from Southern blot analysis. In addition, northern blot analysis indicated expression and proper splicing of the maize ubiquitin gene first intron from the primary chimeric transcript in these transgenic rice plants, and western blot analysis and enzymic assays verified expression of the active *bar* gene product. Apparent mendelian segregation for bialaphos resistance in T_1 progeny of primary transformants was confirmed.

Although the production of transgenic rice plants has been reported by a number of laboratories (1, 4, 6, 13, 17, 19, 24, 26, 28, 30), the regeneration of fertile, stably transformed rice has yet to be made routine and efficient.

In many plant transformation studies, drug resistance genes such as neomycin phosphotransferase or hygromycin phosphotransferase have been used as selectable markers (19, 24, 25, 28). One recent alternative strategy that has emerged is based on the use of marker genes that confer resistance to herbicides (7, 8, 10, 11). Bialaphos and PPT[3] are known to be potent herbicides with a broad spectrum of toxicity to numerous crops as well as weeds. Bialaphos was first registered as a tripeptide antibiotic produced by *Streptomyces hygroscopicus* SF1293. It consists of PPT, an analog of L-glutamic acid, and two L-alanine residues. Upon removal of the alanine residues by endogenous peptidases in plant cells, the resulting PPT inhibits glutamine synthetase, thus causing a rapid accumulation of ammonia that leads to plant cell death (22, 23).

The *bar* gene, cloned from *S. hygroscopicus*, encodes the enzyme PAT, which acetylates the NH_2-terminal group of PPT, abolishing its herbicidal activity (15, 27). Chimeric constructs consisting of the cauliflower mosaic virus 35S promoter fused to the *bar* gene have been transferred into tobacco, tomato, potato (7), and rape (8) through *Agrobacterium*-mediated transformation, and such transgenic plants were resistant to both PPT and bialaphos. Similarly, the same gene has been introduced into the monocot plant, maize, by microprojectile bombardment with the production of fertile, transgenic plants (10, 11). In the case of rice (*Oryza sativa* L.), Dekeyser et al. (9) pointed out the potential usefulness of the *bar* gene as a selectable gene for transformation. More recently, Christou et al. (4) regenerated transgenic rice plants using a cauliflower mosaic virus 35S promoter-*bar* chimeric gene by electric discharge particle acceleration.

Here, we report the electroporation-mediated transformation of rice protoplasts with a chimeric gene consisting of the promoter, first exon, and first intron of the maize polyubiquitin gene (*Ubi-1*) (3) fused to a *bar* gene, and the regeneration of stably transformed transgenic rice plants.

MATERIALS AND METHODS

Plant Materials

Suspension cells derived from callus of germinating seeds of japonica rice (*Oryza sativa* L. var Yamahoushi) were maintained in amino acid medium (29) containing 1 mg/L of casein hydrolysate. The culture was kept on a gyratory shaker (100 rpm) at 25°C and subcultured once a week.

Plasmids

A 2-kb *Hin*dIII-*Bam*HI fragment containing the maize *Ubi-1* promoter, first exon, and first intron (3) was ligated into

[1] This research was supported by grants from the Ministry of Education, Science, and Culture of Japan (H.U.), the Ministry of Agriculture, Forestry and Fishery of Japan (H.U.), Suhara Memorial Foundation (H.U., Se.T.), Torey Science Foundation (H.U.), and the Rockefeller Foundation (H.U.). Work in the laboratory of P.H.Q. was supported by a grant from Agrigenetics Research Associates and U.S. Department of Agriculture/Agricultural Research Service No. 5335-23000-004-00D.

[2] Present address: Department of Biology, George Mason University, Fairfax, VA 22030-4444.

[3] Abbreviations: PPT, phosphinothricin; PAT, phosphinothricin acetyltransferase; PCR, polymerase chain reaction.

the 3.6-kb chimeric *bar* structural gene (15)—nopaline synthase terminator (2)—pUC12 sequence, resulting in the production of pUBA (Fig. 1A). Because the *bar* gene does not contain an ATG initiation codon, synthetic oligonucleotides containing an ATG sequence were added to the 5′ end of the *bar* gene. Details of this vector construction will be reported elsewhere. The plasmid pBR328012 containing the *rolC* gene of *Agrobacterium rhizogenesis* Ri plasmid TL-DNA (21) was also used for cotransformation with pUBA.

Electroporation and Selection of Transformants

Four-day-old suspension-cultured cells were incubated in a solution containing 2.0% Cellulase Onozuka RS, 0.2% Macerozyme R10, 0.1% $CaCl_2 \cdot 2H_2O$, and 6.5% mannitol (pH adjusted to 5.7 with NaOH). After 4 h at 25°C, protoplasts were filtered through nylon mesh (30 μm) and collected by centrifugation (100*g*, 4 min). Protoplasts were washed twice with an electroporation buffer (0.5 mM Mes [pH 5.8], 70 mM KCl, 4 mM $CaCl_2 \cdot 2H_2O$, and 6.5% mannitol) by centrifugation (100*g*, 3 min). To remove cell debris, protoplasts were floated on the surface of 30% (v/v) Percoll in electroporation buffer and collected by centrifugation (100*g*, 5 min). Purified protoplasts were resuspended in electroporation buffer. A mixture of plasmid DNAs, 10 μg each of pUBA and pBR328012 in a 100-μL electroporation buffer, was added to 1 mL of protoplast suspension (5×10^6 cells). This mixture was then subjected to an electrical pulse (375 V/cm, 250 μF, and 200 Ω) using a Gene Pulsar apparatus (Bio-Rad).

Following electroporation, protoplasts were cultured in a medium according to the method of Matsuki et al. (14). After 2 weeks, 2 (or 10) μg/mL (final concentration) of bialaphos was added to the culture medium. Colonies capable of proliferating in the bialaphos medium were transferred to regeneration medium consisting of N6 salts (5), 3% sucrose, 1 mg/L of kinetin, and 1% agarose. Regenerated plants were saved for further analysis.

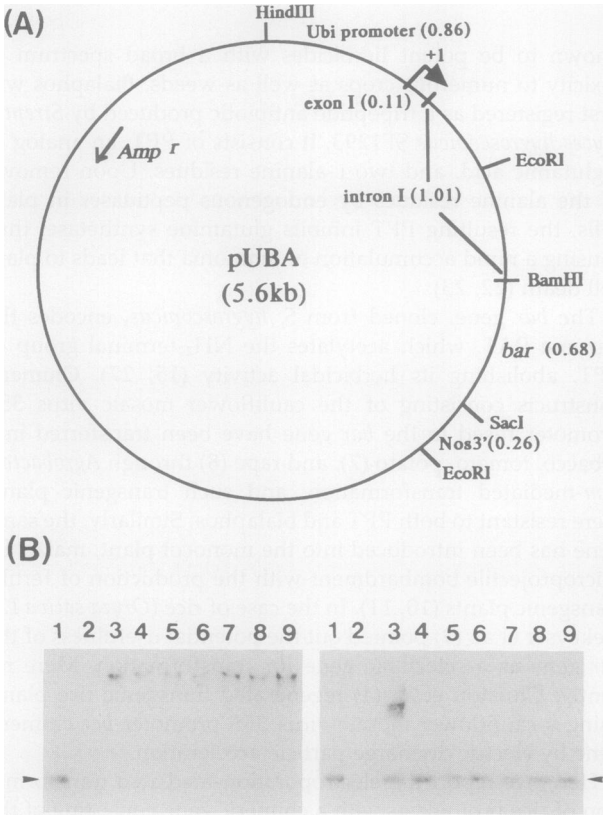

Figure 1. A, Chimeric plasmid pUBA used for rice transformation. *Ubi-1* promoter/exon I/intron I, Fragment from the maize ubiquitin gene *Ubi-1* containing the promoter, first exon, and first intron (3); *bar*, bialaphos resistance gene (15); Nos3′, nopaline synthase terminator (2). The chimeric *Ubi-1-bar* gene was cloned into the *Hin*dIII/*Eco*RI sites of pUC12. Numerals indicate kb. B, Southern blot analysis of seven transgenic T_0 plants. Left, The results for undigested DNA; right, the results for DNA digested with *Bam*HI and *Sac*I, which cut within the chimeric gene releasing the *bar*-coding region. For each plant, 5 μg of total DNA was digested (or not) with restriction enzymes, subjected to gel electrophoresis, blotted onto Hybond N+, and hybridized with a probe representing the *bar*-coding region. Lane 1, One copy marker reconstructed from pUBA digested with *Bam*HI and *Sac*I; lane 2, DNA from an untransformed rice plant; lanes 3 to 9, DNA from primary transformants. Arrow indicates 0.68 kb band. λ-DNA digested with *Hin*dIII was used as a size marker.

Southern Blot Analysis

Total DNA was isolated from leaves of rice plants according to the method of Shure et al. (20). DNA (5 μg) was digested with restriction enzymes and electrophoresed in a 0.8% agarose gel. DNA fragments were blotted to nylon membrane (Hybond-N+; Amersham), followed by hybridization with probe DNA, which had been labeled with [^{32}P]dCTP using the Multiprime DNA labeling system (Amersham). A *bar* structural gene fragment (0.68 kb) and the promoter/first exon/first intron region (2 kb) of the maize ubiquitin gene were used as probes.

PCR Analysis

5′-GGATCCATGAGCCCAGAA-3′ (18-mer) and 5′-TCAGATCTCGGTGACGGGCA-3′ (20-mer), representing sequences on the *bar* gene, were used as primers. Total DNA (1 μg) was subjected to 30 cycles of amplification of three steps each (94°C, 1 min; 60°C, 2 min; 72°C, 2 min) in PCR buffer (10 mM Tris-HCl [pH 8.4], 50 mM KCl, 1.5 mM MgCl2, and 0.01% [w/v] gelatin) containing 0.2 mM each dNTP, 1 μM each primer, and 2.5 units of Ampli Taq (Takara Co. Ltd.). PCR products were then analyzed by gel electrophoresis.

Northern Blot Analysis

Total RNA (20 μg) was electrophoresed on a formaldehyde-agarose gel, blotted onto a nylon membrane (Hybond-N+; Amersham), and hybridized with a *bar* gene fragment (0.68 kb).

Immunological Methods

PAT antiserum was prepared from PAT-immune rabbits. Peroxidase-labeled anti-PAT-Fab′ was prepared according to

the procedure of Hashida et al. (12). Crude cell extracts (100 μg of protein) were used to precipitate PAT using PAT antiserum. Resulting pellets were dissolved in 10 μL of sample buffer (0.01 M Tris-HCl [pH 9.0], 4% SDS, 30% sucrose, 2% mercaptoethanol, and 0.001% bromophenol blue) and subjected to SDS-PAGE. Following electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose filter. The filter was sequentially treated with peroxidase-labeled anti-PAT-Fab' and developed by Konica immunostain horseradish peroxidase (Konica Co., Tokyo, Japan).

PAT Enzymic Activity Assay

The PAT assay was performed as described by De Block et al. (7).

RESULTS AND DISCUSSION

In three independent experiments, a total of 158 colonies resistant to 2 (or 10) mg/L of bialaphos were obtained from DNA-treated protoplasts. No colonies were recovered from protoplasts not treated with DNA under the same selection conditions. Subsequently, selected calli were transferred to regeneration medium. Ultimately, 10 independent bialaphos-resistant plants were transferred to soil and grown in the greenhouse. After about 2 months, untransformed control and transgenic plants were sprayed with a 1% Basta (20% ammonium salts of PPT)-0.1% Tween 20 mixture. Discoloration of leaves of control plants was seen as early as the third day, whereas transgenic plants displayed a normal appearance (Fig. 2A).

When DNA samples from the bialaphos-resistant primary transformant plants (T_0) were digested with *Bam*HI plus *Sac*I and hybridized with a probe representing the *bar*-coding region, the presence of an intact *bar*-coding region (0.68 kb) was detected in transformed plants (Fig. 1B, right, lanes 3–9). This band was not detected in untransformed plants (Fig. 1B, right, lane 2). Evidence that the *bar* sequence is integrated into the genomic DNA of the rice cells is provided by the comigration of the *bar*-hybridizable band with the high mol wt (>40 kb) fragments of ethidium bromide-stainable DNA present in undigested samples (Fig. 1B, left, lanes 3–9). No hybridizable band was present in DNA from untransformed plants (Fig. 1B, left, lane 2). Data from PCR analysis of seven transgenic plants are also consistent with the presence of the *bar* structural gene (data not presented). Furthermore, DNA of a primary transgenic plant digested with *Hin*dIII plus *Bam*HI and hybridized with the 2.0-kb *Hin*dIII-*Bam*HI fragment of pUBA (pro/int probe, Fig. 1A) yielded a 2.0-kb band. On the other hand, in DNA digested with *Bam*HI alone and probed with the 0.68-kb *bar*-coding region fragment, a 2.6-kb band was observed. Because pUBA contains an unique *Bam*HI site that would produce a 5.6-kb linear DNA fragment, the 2.6-kb band presumably represents a border fragment resulting from *Bam*HI-catalyzed cleavage within the original plasmid sequence and at an adjacent site in the flanking host DNA. Both probes were used separately, and DNA cut with *Hin*dIII alone produced an identical 6.2-kb fragment. Because pUBA contains a unique *Hin*dIII site that would produce a linear fragment of 5.6 kb from the original plasmid, the 6.2-kb fragment presumably contains the colinear *Ubi-1-bar* sequence plus flanking host DNA that has been cut at a *Hin*dIII site. This result is consistent with integration of an intact *Ubi-1-bar* chimeric gene into the rice genome (data not presented).

To examine the transcription of the introduced foreign gene in primary transformants, total RNA was extracted from

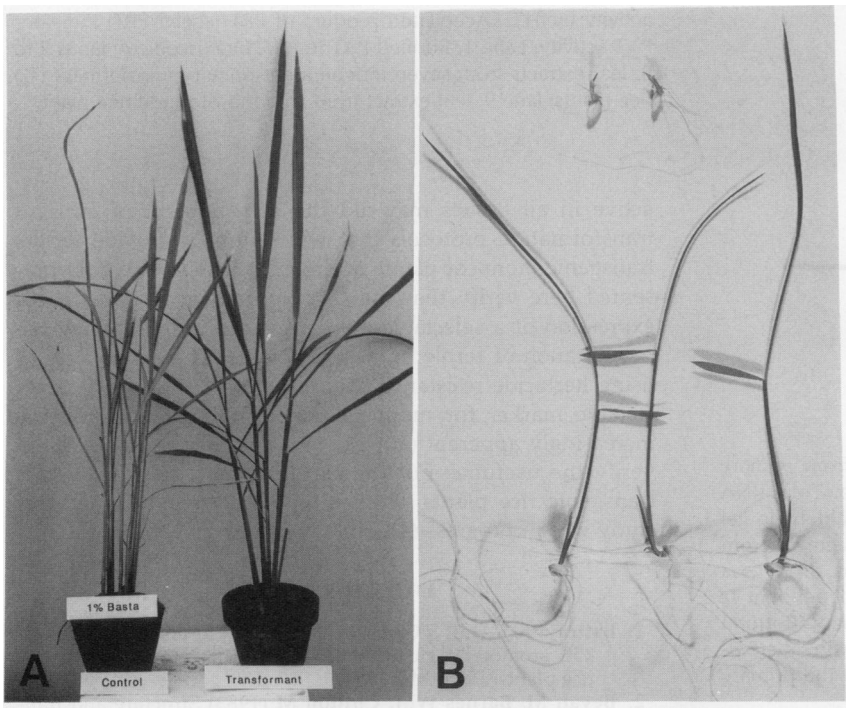

Figure 2. A, Basta resistance in transgenic rice plants. The herbicide formulation, Basta, contains 200 mg/L of glufosinate, the ammonium salt of PPT. Plants (about 3 months old) were sprayed with a 1% Basta solution (v/v) containing 0.1% (v/v) Tween 20. Photographic observations were recorded 2 weeks after the Basta treatment. B, Segregation of bialaphos-resistant and bialaphos-susceptible plants in the progeny (T_1 generation) of selfed primary transformant (T_0 generation) plants. Selection was made 7 d after germination in the presence of 50 mg/L of bialaphos.

seven bialaphos-resistant plants, and *bar* mRNA was analyzed by northern blot using a *bar*-coding region as a probe. In transgenic plants, a 0.8-kb *bar* mRNA was detected, indicating expression of the chimeric *Ubi-bar* gene as well as correct splicing of the ubiquitin intron sequences in the rice plants (Fig. 3, lanes 1–7). No *bar* mRNA was detected in nontransformed controls (Fig. 3, lane 8).

The expression of PAT was determined in the seven bialaphos-resistant plants examined as described above. Analysis of crude leaf extracts by western blotting revealed the presence in transgenic plants (Fig. 4A, lanes 2–8) of a polypeptide (23 kD) that comigrated with PAT purified from *S. hygroscopicus* (lane 10). No immunoreactive band was detected in untransformed control plants (Fig. 4A, lane 9). The results of PAT enzymic activity assays are shown in Figure 4B. After separation by TLC, ^{14}C-labeled acetyl PPT was seen only in transgenic plants (Fig. 4B, lanes 2–8) and not in untransformed plants (Fig. 4B, lane 9).

To examine the inheritance of the introduced foreign genes, seeds (T_1 generation) of transgenic plants were disinfected and allowed to germinate in a liquid Murashige-Skoog medium (16). After 7 d, seedlings were transferred to the same medium containing 50 mg/L of bialaphos. The number of plants resistant or sensitive to the drug was recorded after 2 weeks (Fig. 2B). The ratio of bialaphos-resistant to bialaphos-sensitive seedlings is consistent with a 3:1 segregation ratio. Moreover, we were able to detect PAT protein and PAT enzymic activity in such bialaphos-resistant T_1 plants. These results verify transmission of the introduced *Ubi-bar* gene to the progeny of the primary transgenic rice plants.

The availability of strong monocot promoters that are active in all tissues may aid the development of efficient transformation protocols that will routinely provide fertile, transgenic monocot plants at high frequency. The data presented here verify that the *Ubi-1* promoter drives efficient expression of a selectable marker gene in rice, allowing the regeneration of fertile, transgenic plants. The advantages of using herbicide resistance over antibiotic resistance as a selectable marker for monocot transformation have become increasingly apparent (10, 11, 18). The data presented here verify the usefulness of the *bar* gene for producing fertile, transgenic rice plants, as was reported recently while this study was in progress (4).

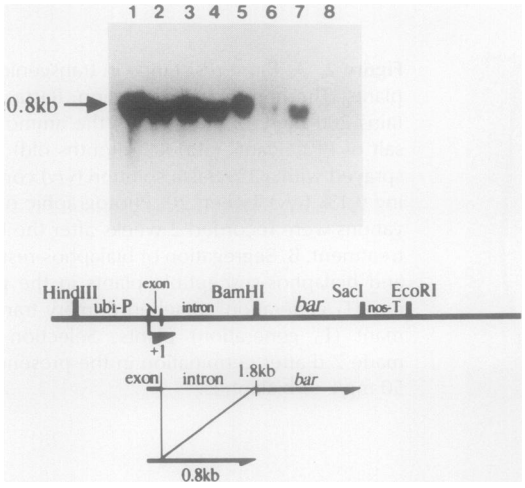

Figure 3. Northern blot analysis of RNA extracted from primary transformant (T_0) and untransformed rice plants. Top, Total RNA (20 μg) was electrophoresed in a 1.6% agarose-formaldehyde gel and blotted to a nylon membrane. A *Bam*HI/*Sac*I fragment from pUBA representing the *bar* gene-coding region was used as a hybridization probe. Lanes 1 to 7, RNA from seven independent T_0 plants; lane 8, RNA from an untransformed control plant. Bottom, Map of chimeric *Ubi-1-bar* gene and a schematic showing the 0.8-kb product expected as a result of correct splicing of the primary chimeric transcript.

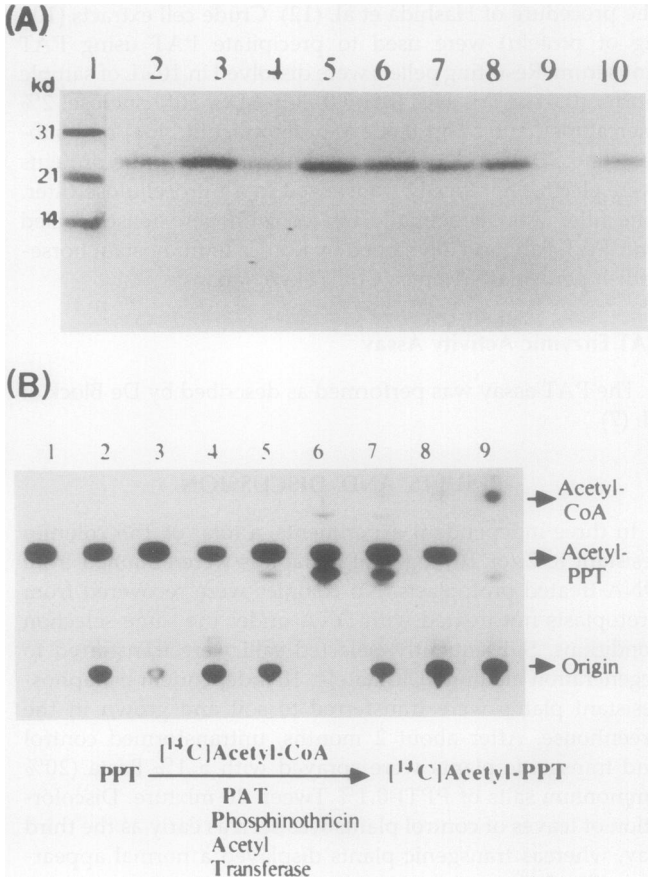

Figure 4. A, Detection of PAT protein by immunoblotting of leaf extracts from primary transformant (T_0) rice plants. Lane 1, Molecular mass markers; lanes 2 to 8, extracts from seven independent T_0 plants; lane 9, extract from an untransformed plant; lane 10, 30 ng of purified PAT from *S. hygroscopicus*. B, Detection of PAT activity by TLC. Acetylated product of PPT (acetyl PPT) indicates PAT activity. Lane 1, Purified PAT from *S. hygroscopicus*; lanes 2 to 8, leaf extracts from seven independent primary transformant (T_0) rice plants; lane 9, leaf extract from an untransformed rice plant.

LITERATURE CITED

1. **Battraw MJ, Hall TC** (1990) Histochemical analysis of CaMV 35S promoter-β-glucuronidase gene expression in transgenic rice plants. Plant Mol Biol **15:** 527–538
2. **Bevan M, Barnes WM, Chilton M** (1983) Structure and tran-

scription of the nopaline synthase gene region of T-DNA. Nucleic Acids Res **1:** 369–385

3. **Christensen AH, Sharrock RA, Quail PH** (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol **18:** 675–689
4. **Christou P, Tameria LF, Kofron M** (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varietes via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technol **19:** 957–962
5. **Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY** (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin **18:** 659–668
6. **Datta SK, Peterhans A, Datta K, Potrykus I** (1990) Genetically engineered fertile indica-rice recovered from protoplasts. Bio/Technol **8:** 736–740
7. **De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gossele V, Movva NR, Thompson C, Van Montagu M, Leemans J** (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J **6:** 2513–2518
8. **De Block M, De Brouwer D,, Tenning P** (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. Plant Physiol **91:** 694–704
9. **Dekeyser R, Claes B, Marichal M, Van Montagu M, Caplan A** (1989) Evaluation of selectable markers for rice transformation. Plant Physiol **90:** 217–223
10. **Fromm ME, Morrish F, Armstrong C, Williams R, Thomas J, Klein TM** (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technol **8:** 833–839
11. **Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR Jr, Willetls NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG** (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell **2:** 603–618
12. **Hashida S, Imagawa M, Inoue S, Ruan KH, Ishikawa E** (1984) More useful maleimide compounds for the conjugation of Fab' to horseradish peroxidase through thiol groups in the hinge. J Appl Biochem **6:** 56–63
13. **Hayashimoto A, Li Z, Murai N** (1990) A PEG-mediated protoplast transformation system for production of fertile transgenic rice plants. Plant Physiol **93:** 857–863
14. **Matsuki R, Onodera H, Yamauchi T, Uchimiya H** (1989) Tissue-specific expression of the *rolC* promoter of Ri plasmid in transgenic rice plants. Mol Gen Genet **220:** 12–16
15. **Murakami T, Anzai H, Imai S, Satoh A, Nagaoka K, Thompson CJ** (1986) The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. Mol Gen Genet **205:** 42–50
16. **Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497
17. **Peng J, Lyznik LA, Lee L, Hodges TK** (1990) Co-transformation of indica rice protoplasts with *gus*A and *neo* genes. Plant Cell Rep **9:** 168–172
18. **Potrykus I** (1991) Gene transfer to plants: assessment of published approaches and results. Annu Rev Plant Physiol Plant Mol Biol **42:** 205–225
19. **Shimamoto K, Terada R, Izawa T, Fujimoto H** (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. Nature **338:** 274–277
20. **Shure M, Wessler S, Fedoroff N** (1983) Molecular identification and isolation of *waxy* locus in maize. Cell **35:** 225–233
21. **Sugaya S, Hayakawa K, Handa T, Uchimiya H** (1989) Cell specific expression of the *rolC* gene of the TL-DNA of Ri plasmid in transgenic tobacco plants. Plant Cell Physiol **30:** 649–653
22. **Tachibana K, Watanabe T, Sekizawa Y, Takematsu T** (1986) Inhibition of glutamine synthetase and quantitative changes of free amino acids in shoots of bialaphos-treated Japanese barnyard millet. J Pesticide Sci **11:** 27–31
23. **Tachibana K, Watanabe T, Sekizawa Y, Takematsu T** (1986) Accumulation of ammonia in plants treated with bialaphos. J Pestic Sci **11:** 33–37
24. **Tada Y, Sakamoto M, Fujimura T** (1990) Efficient gene introduction into rice by electroparation and analysis of transgenic plants: use of electroporation buffer lacking chloride ions. Theor Appl Genet **80:** 475–480
25. **Tada Y, Sakamoto M, Matsuoka M, Fujimura T** (1991) Expression of a monocot LHCP promoter in transgenic rice. EMBO J **7:** 1803–1808
26. **Terada R, Shimamoto K** (1990) Expression of CaMV35S-GUS gene in transgenic rice plants. Mol Gen Genet **220:** 389–392
27. **Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J** (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. EMBO J **6:** 2519–2523
28. **Toriyama K, Arimoto Y, Uchimiya H, Hinata K** (1988) Transgenic rice plants after direct gene transfer into protoplasts. Bio/Technol **6:** 1072–1074
29. **Toriyama K, Hirata K** (1985) Cell suspension and protoplast culture in rice. Plant Sci **41:** 179–183
30. **Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC, Davey MR** (1988) Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep **7:** 379–384